Volume 296, number 3, 249–253 FEBS 10604 January 1992

Proton NMR study of the interaction of benzo(a)pyrene with rat liver microsomal cytochrome P-450

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Received 28 October 1991

^{1}H nuclear magnetic resonance longitudinal relaxation time (T_1) measurements were used to study the interaction of 1,3,6-H-benzo(a)pyrene (1,3,6-H-BaP) with microsomal cytochrome P-450 from livers of phenobarbital- and β-naphthoflavone-treated rats. Upon addition of various amounts of cytochrome P-450 to solutions of BaP, the T_1 values for the three BaP protons decreased. When the hemeprotein was converted to its carbon monoxyferrous derivative in the presence of BaP, the T_1 values of the BaP protons increased. The paramagnetic contributions to the observed T_1 values were calculated and resulted in distance estimates of >6 Å for the three BaP protons in both cases.

Cytochrome P-450; Benzo(a)pyrene; ^{1}H NMR; Relaxation measurement

1. INTRODUCTION

The cytochrome P-450 (P-450) enzyme system, which catalyzes the oxidation of a wide variety of chemical agents, serves as the initial biological receptor for polycyclic aromatic hydrocarbons like benzo(a)pyrene (BaP) [1]. Cytochrome P-450, along with molecular O_2 and coenzymes NADPH and NADPH cytochrome *c* reductase catalyze the formation of arene oxides from BaP [2]. The BaP oxides are further metabolized to produce three classes of products: BaP phenols, BaP dihydrodiols and BaP quinones [1]. The dihydrodiols undergo further oxidative metabolism, catalyzed by P-450, to the highly reactive diol-epoxides which are thought to be the 'ultimate' carcinogen of BaP metabolism [3]. The initial step in this overall process involves the formation of a complex between BaP and cytochrome P-450. The purpose of the present work was to obtain information on the nature of interaction of benzo(a)pyrene with its metabolizing enzyme cytochrome P-450 using nuclear magnetic resonance spectroscopy.

The NMR method has been used by Novak et al. [4–6] to study the interaction of cytochrome P-450 with substrates such as acetanilide and xylidine, and by Woldman et al. [7,8] with ligands such as aminopyrine and 4-methoxypyridine. The method is based on the fact that rapid relaxation of the electron spin of Fe(III) in the active center of cytochrome P-450 enhances the relaxation of substrate protons in an enzyme–substrate complex according to the Solomon-Bloembergen equation [9]:

$$\frac{1}{T_{1,M}} = \frac{2S(S+1)}{15 r^6} \cdot \left(\frac{h\gamma g\beta}{2\pi}\right)^2 \cdot \left[\frac{3\tau_C}{1+\omega_I^2\tau_C^2} + \frac{7\tau_C}{1+\omega_S^2\tau_C^2}\right] \quad (1)$$

In this equation, $T_{1,M}$ is the relaxation time of a nucleus bound in the proximity of a paramagnetic metal ion; r is the average electronnuclear distance; τ_c is the dipolar correlation time; ω_I and ω_S are the nuclear and electron resonance frequencies and S is the total electron spin. The constants can all be gathered together under one term and equation (1) reduces to:

$$r = (\text{constant})\,[T_{1,M} \cdot f(\tau_C)]^{1/6} \quad (2)$$

where $f(\tau_C)$ refers to the expression in brackets shown in equation (1). For a nucleus of the substrate which forms a complex with the paramagnetic ion, the contribution to the observed longitudinal relaxation rate due to the paramagnetic ion, $T_{1,p}^{-1}$ is given by equation (3):

$$\frac{1}{T_{1,p}} = \frac{1}{T_{1,obs}} - \frac{1}{T_{1,0}} \quad (3)$$

where $T_{1,0}$ is the relaxation time in the absence of paramagnetism and $T_{1,obs}$ is the relaxation time in the presence of the hemeprotein. $T_{1,M}$ is related to the residence time of the substrate in the complex, τ_M and the molar fraction of the complex α_M [10]:

$$\frac{1}{T_{1,p}} = \frac{\alpha_M}{T_{1,M}+\tau_M} \quad (4)$$

Under conditions where $\tau_M < T_{1,M}$, fast exchange prevails and $T_{1,M} = \alpha_M \cdot T_{1,p}$. Therefore, the parameter r, which is the distance between the paramagnetic ion

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and the relaxing nucleus can be calculated using equation (1). In the present work, the NMR technique of longitudinal relaxation time measurement has been employed to study the interaction of 1,3,6-H-BaP with cytochrome P-450 from liver microsomes of phenobarbital- and beta-naphthoflavone-treated rats.

2. MATERIALS AND METHODS

In our experiments, deuterated solvents were obtained from either Aldrich Chemical Co. or Cambridge Isotope Laboratories and were used unpurified. 1,3,6-H-BaP was prepared following a modified procedure for the preparation of 1,3,6-D-BaP described by Cavalieri and Calvin [11]. 5 mg of perdeuterated BaP was dissolved in concentrated H_2SO_4 (0.75 ml) at 4°C and left for 6 min. The red solution was poured in 5 ml H_2O and 2.5 ml $CHCl_3$ at 4°C. The organic layer was separated and the aqueous layer was extracted again with cold $CHCl_3$ (2.5 ml). The total organic solution was washed with cold H_2O (5 ml), dried with Na_2SO_4 and evaporated. The product obtained was dissolved in a solvent mixture containing 50% dioxane-d_8, 30% D_2O and 20% glycerol-d_8 for NMR studies.

The induction of male, white Sprague–Dawley rats (175–200 g) by phenobarbital (PB) and β-naphthoflavone (βNF) as well as the isolation of microsomes were carried out using standard methods [12,13]. The protein concentrations were determined with a Pierce BCA protein assay kit, using bovine serum albumin as the standard [14]. The cytochrome P-450 concentration was determined from its absorbance at 450 nm using a molar absorptivity constant of 91 $mM^{-1} \cdot cm^{-1}$ [15]. The content of cytochrome P-450 in our preparation ranged from 1.3 to 1.8 nmol of P-450 per mg protein. The microsomes used for ^1H NMR studies were stored in buffers made up in 100% D_2O and deuterated glycerol to avoid strong solvent proton peaks.

All solvents used for NMR experiments were treated with Chelex-100 (Bio-Rad) to remove contaminating paramagnetic ions. The solutions were deoxygenated by bubbling N_2 gas through the solution for 10 min, prior to all NMR experiments.

Optical measurements were made using a Hewlett-Packard 8451A diode array spectrophotometer with a built-in microprocessor. NMR spectra were recorded using a Varian VXR-400S NMR spectrometer, equipped with a 9.4 T, 54 mm Oxford superconducting magnet. The longitudinal relaxation time (T_1) was determined using the inversion-recovery sequence (180° x — t — 90° x — acquisition — D) [16,17].

3. RESULTS

The ^1H FT NMR spectrum of 1,3,6-H-BaP in 50% dioxane-d_8, 20% glycerol-d_8 and 30% D_2O is shown in the last scan of the sequence presented in Fig. 1. The proton spectrum of this compound consists of three signals: a singlet at δ = 8.62 ppm indicative of the proton at position 6, another singlet at δ = 8.31 ppm corresponding to the proton at position 1, and another singlet at δ = 8.17 ppm ascribable to the proton at position 3. These signals are well-defined and relatively sharp singlets ideally suited for monitoring intermolecular interactions by the longitudinal relaxation time technique [6].

A typical 180° — t — 90° inversion recovery sequence for 1,3,6-H-BaP, consisting of partially relaxed FT NMR spectra employed in the calculation of T_1 relaxation times, is also depicted in Fig. 1. This stacked plot represents the peak amplitude (either positive or negative) obtained at a particular value of delay time t

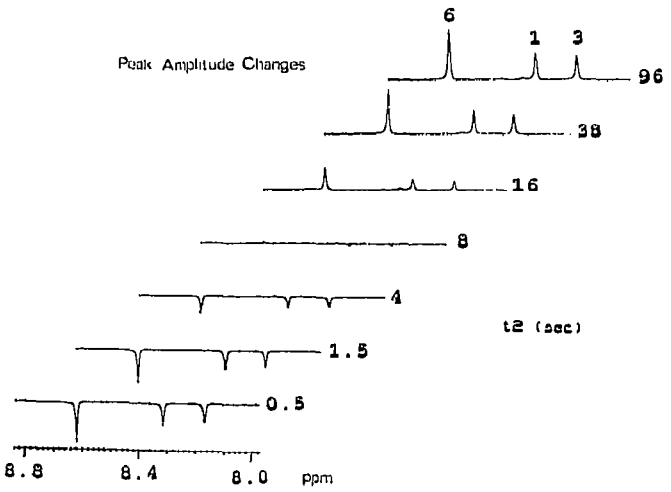

Fig. 1. ^1H FT NMR spectrum and inversion-recovery sequence spectrum of 1,3,6-H-BaP. A solution of 1,3,6-H-BaP (9.05 mM) in 50% dioxane-d_8, 20% glycerol-d_8 and 30% D_2O was prepared (total volume, 0.6 ml), and the spectrum was recorded at 20°C. The chemical shifts of these protons are at 8.62, 8.31 and 8.17 ppm corresponding to the protons at positions 6, 1 and 3 respectively. These chemical shifts are referenced from the H_2O-soluble TMS-equivalent DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate).

in the inversion recovery pulse sequence. When the intensity of each peak is plotted as a function of delay time t, the resulting plot is presented in Fig. 2, with a theoretical best fit curve connecting the experimental points to obtain the value of T_1.

The addition of a hemeprotein such as cytochrome P-450 to a solution of 1,3,6-H-BaP should result in an

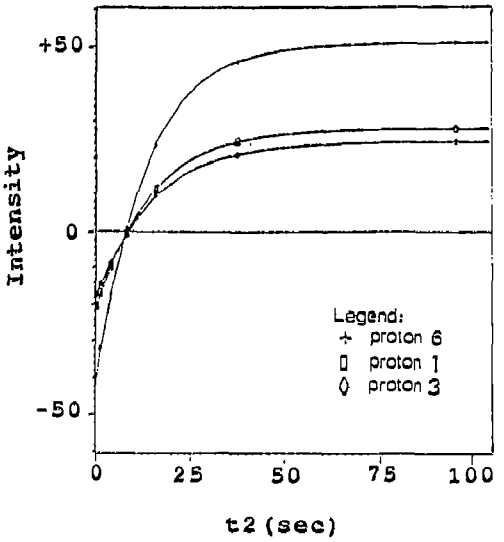

Fig. 2. The magnetization recovery of 9.05 mM 1,3,6-H-BaP as a function of delay time t. The intensity of each peak in the inversion recovery sequence spectrum is plotted as a function of delay time t, with a theoretical best fit curve connecting the experimental points, to get the best values of T_1.

Table I

Effects of microsomal cytochrome P-450 (PB-induced) on T_1 values of 1,3,6-H-BaP

^{1}H Position	Cytochrome P-450 (μM)	1,3,6-H-BaP (mM)	$T_{1,blank}$[a] (s)	$T_{1,obs}$[b] (s)	$T_{1,P\text{-}450+2\text{-}CO}$[c] (s)
6	5.45	9.05	12.98±0.16	3.07±0.05	7.99±0.35
1	5.45	9.05	14.18±0.10	4.51±0.18	8.51±0.19
3	5.45	9.05	14.79±0.13	4.98±0.18	9.05±0.23
6	10.13	9.13	11.56±0.13	1.67±0.07	2.86±0.15
1	10.13	9.13	12.40±0.07	2.16±0.17	2.87±0.44
3	10.13	9.13	12.44±0.06	6.60±0.63	7.68±0.65
6	15.65	9.13	13.21±0.26	1.73±0.11	2.96±0.42
1	15.65	9.13	15.36±0.10	1.99±0.17	3.13±0.42
3	15.65	9.13	15.88±0.04	3.02±0.11	3.03±0.35
6	20.85	9.05	14.91±0.15	1.92±0.13	4.05±0.43
1	20.85	9.05	16.15±0.08	2.51±0.24	3.41±0.56
3	20.85	9.05	16.83±0.07	3.02±0.29	3.89±0.61

For definitions of a, b and c, see footnotes to Table II.

increased relaxation rate of the three protons, since under this condition, interactions are expected to occur between the substrate and the paramagnetic center of the P-450 [6]. As shown in Table I under $T_{1,obs}$, the addition of a 5.45 μM solution of rat liver microsomal cytochrome P-450 to about 9.05 mM 1,3,6-H-BaP produced a decrease in the relaxation times of the three BaP protons.

In order to evaluate the paramagnetic contribution to the observed relaxation rate increase, the cytochrome P-450 was converted to the diamagnetic ferrous carbonyl complex in situ, and the relaxation times were again determined (see Table I under $T_{1,P\text{-}450+2\text{-}CO}$). These measurements show that formation of the ferrous-carbonyl form of the hemeprotein resulted in an increase in the T_1 values observed for 1,3,6-H-BaP.

Table I summarizes the relaxation times obtained for the 1,3,6-H-BaP protons in the absence and presence of varying amounts of oxidized and reduced phenobarbital-induced rat liver microsomal cytochrome P-450. The relaxation times measured correspond to $T_{1,blank}$ in the absence of P-450, $T_{1,obs}$ in the presence of oxidized P-450 and $T_{1,P\text{-}450+2\text{-}CO}$ in the presence of reduced P-450. These results show that the addition of aliquots of solutions of microsomal cytochrome P-450 to a solution of 1,3,6-H-BaP produced marked decreases in the ^{1}H T_1 relaxation times of the three protons. Formation of the carbon monoxyferrous derivative on the other hand, resulted in increased T_1 values observed for 1,3,6-H-BaP.

Similar results were obtained when solutions of β-naphthoflavone-induced microsomal cytochrome P-450 were added to a solution of 1,3,6-H-BaP. The complete results of the longitudinal relaxation time measurements for the interaction of 1,3,6-H-BaP with β-naphthoflavone-induced cytochrome P-450 are given in Table II.

4. DISCUSSION

The results obtained from these studies indicated that the interaction of BaP with microsomal rat liver cytochrome P-450 gave rise to differential paramagnetic rate changes that are observed by NMR. The estimation of the substrate-heme iron atom distances is based on

Table II

Effects of microsomal cytochrome P-448 (BNF-induced) on T_1 values of 1,3,6-H-BaP

^{1}H Position	Cytochrome P-448 (μM)	1,3,6-H-BaP (mM)	$T_{1,blank}$[a] (s)	$T_{1,obs}$[b] (s)	$T_{1,P\text{-}450+2\text{-}CO}$[c] (s)
6	2.88	12.5	12.88±0.46	2.97±0.08	7.19±0.57
1	2.88	12.5	14.11±0.15	4.66±0.19	7.99±0.47
3	2.88	12.5	14.67±0.09	5.04±0.19	8.89±0.36
6	4.53	13.5	11.34±0.39	2.69±0.07	5.34±0.59
1	4.53	13.5	12.54±0.07	4.05±0.24	5.28±0.45
3	4.53	13.5	13.26±0.09	3.51±0.30	5.94±0.43
6	7.25	13.0	12.59±0.28	1.01±0.22	3.09±0.17
1	7.25	13.0	13.49±0.24	1.09±0.28	3.35±0.75
3	7.25	13.0	13.91±0.34	1.07±0.29	3.95±0.49

[a] $T_{1,blank}$ = T_1 value of 1,3,6-H-BaP proton peaks in the total absence of microsomal P-450/P-448.
[b] $T_{1,obs}$ = T_1 value of 1,3,6-H-BaP proton peaks in the presence of microsomal P-450/P-448.
[c] $T_{1,P\text{-}450+2\text{-}CO}$ = T_1 value of 1,3,6-H-BaP proton peaks in the presence of the reduced-CO (diamagnetic) form of the microsomal P-450/P-448.

the use of equations 2, 3 and 4 and under the assumption that rapid exchange prevailed (i.e. $T_{1M} \approx \alpha_M \cdot T_{1,p}$). This corresponds to a situation where BaP is coming on and off the enzyme's active site, such that the lifetime of the enzyme–substrate complex is considered to be in the fast-exchange range. It is unlikely that the system is in the region of slow exchange since the substrate is several orders of magnitude more concentrated than the enzyme (i.e substrate to hemeprotein ratio is 1000:1) [4]. Under this condition, less than 1.0% of the substrate would be fixed in complex and would not be expected to contribute to any marked changes in T_1 of the free substrate signal. Hence, the assumption of rapid exchange for BaP-cytochrome P-450 appears valid.

The other assumption used in these calculations was that the liver microsomal P-450^{+2}–CO is diamagnetic. It has been shown previously by several investigators that the reduced forms of other hemeproteins such as myoglobin and hemoglobin are diamagnetic [18], and that the reduced-CO form of the camphor-induced cytochrome P-450 from *Pseudomonas putida* has also been judged to be diamagnetic [19]. Hence, the evaluation of diamagnetic protein interactions upon the T_1 of the substrate can be established by changing the hemeproteins to the carbon monoxyferrous form in situ and comparing the T_1 values in the presence of these diamagnetic derivatives with the T_1 values in the absence of hemeproteins.

The third assumption used in these distance calculations was that the dipolar interaction was the primary relaxation mechanism for these NMR longitudinal relaxation time measurements [16]. This arises from the coupling of nuclear and electronic magnetic moments which provide efficient relaxation pathways. This means that the longitudinal relaxation rate T_{1M}^{-1} of the BaP nucleus is enhanced due to its coupling with the unpaired electrons of the ferric iron of cytochrome P-450.

The paramagnetic contributions of PB- as well as βNF-induced microsomal cytochrome P-450 to the relaxation rates, $T_{1,p}^{-1}$, of 1,3,6-H-BaP were then evaluated using the data presented in Tables I and II and using equation 3. The results were used to calculate the parameter $T_{1,M}$ which is the relaxation time of the BaP nucleus bound in the proximity of the heme iron. These relaxation times and the estimated value of the correlation time of 2.82×10^{-10} s for high-spin Fe(III) [20] were used to calculate values of r, which correspond to the distances of the three BaP nuclei from the heme iron. The results are given in Table III. These values are the average Fe-BaP proton distances and are obtained from the same batch of microsomal preparations.

The distances determined between the BaP protons and P-450 iron were all greater than 6 Å, with the sixth proton of BaP having a closer approach to the heme iron relative to the other two protons. This is observed for both the PB-induced as well as the βNF-induced

Table III

Estimated benzo(a)pyrene-cytochrome P-450 distances

The distances of approach of the 1, 3 and 6 protons of BaP to the paramagnetic heme iron of cytochrome P-450 using an estimated value of $f(\tau_C) \approx 2.82 \times 10^{-10}$ for high-spin Fe^{+3}.

Inducer	P-450 Isozyme	Proton position	$r_{(Fe-H)}$ (Å); $S = 5/2$[a]
PB	P-450	6	8.72 ± 0.68
		1	9.75 ± 0.93
		3	11.13 ± 1.91
βNF	P-448	6	6.79 ± 0.44
		1	7.66 ± 1.18
		3	7.31 ± 0.82

[a]Distances were calculated using equations 1, 2, 3 and 4 under the assumption that rapid exchange prevailed (i.e. $T_{1M} \approx \alpha_M \cdot T_{1p}$). These distances are the average values ±S.D. and were calculated from paramagnetic relaxation rate values obtained at the concentrations of P-450 indicated in Tables I and II.

microsomal cytochrome P-450 interacting with 1,3,6-H-BaP. The results also showed that the distances obtained for the βNF-induced isozyme, are in general, shorter than those obtained for the PB-induced isozyme. This may indicate that the βNF-induced rat liver isozyme has a higher substrate preference for BaP since the distances obtained indicate that BaP protons are in closer proximity to the heme iron of this isozyme compared to the PB-inducible form. This is in agreement with the findings of Saito and Strobel that the cytochrome P-450 isozyme purified from βNF-pretreated rat liver microsomes has the highest turnover number for BaP [13]. However, a more detailed analysis of its metabolic profile is necessary in order to confirm this result.

In order to investigate whether these differential relaxation rate changes resulted from substrate interaction with the paramagnetic center of the enzyme, coronene, a non-substrate compound, was added to a microsomal solution of cytochrome P-450. The ^{1}H NMR spectrum of coronene at 400 MHz shows a prominent peak at $\delta = 8.15$ ppm. The relaxation time of the coronene peak was measured at 10.19 ± 0.05 s in the total absence of hemeprotein, 6.63 ± 0.24 s in the presence of 20.25 μM PB-induced cytochrome P-450 and 6.85 ± 0.27 s in the presence of the reduced-CO form of the enzyme. The paramagnetic effect calculated from these relaxation time changes corresponds to about 0.005 s which is negligible compared to the paramagnetic relaxation rates obtained for 1,3,6-H-BaP. Hence this supports our assumption that the observed changes in T_1 for 1,3,6-H-BaP are associated with the paramagnetic center of the heme.

The main conclusion that can be drawn from these results is that a considerable distance exists between the P-450 heme iron and the substrate binding site of BaP ($r > 6$ Å). It has been shown previously from ultraviolet

difference spectroscopy that BaP can be classified as a Type I substrate for P-450 [21]. Type I substrates are thought to bind in the vicinity of the heme through a protein binding cavity, while Type II substrates are thought to coordinate directly to the heme iron atom [4]. Since BaP is a Type I substrate, then it is reasonable to suggest that cytochrome P-450 possesses a binding site which is distant from the heme site. If more information on the spin state, dissociation constant and correlation time for the BaP-P-450 complex becomes available, the spatial orientation of the bound substrate with respect to the heme iron atom may be more clearly defined.

Acknowledgements: The authors thank Drs. C. Sultany and J.A. Butcher for their help with the NMR work.

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